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14. ABSTRACT

Prostate cancer is the second leading cause of cancer death in men in the United States. A few epidemiology studies have indicated that exposure to PhIP, a rodent prostate carcinogen formed in meat during cooking, may be an important risk factor for prostate cancer in humans. Therefore, a highly sensitive biomarker assay is urgently needed to clarify the role of PhIP in prostate cancer. The goal of this project is to develop an assay that can be used to more accurately quantify human exposure to PhIP and potential prostate cancer risk. Our hypothesis is that an Accelerator Mass Spectrometry-based method can be developed to measure protein adducts of PhIP in the blood of humans. This will provide a measure of the internal dose, as well as the capacity for carcinogen bioactivation to a form that can initiate the cancer process. Towards this goal, we have characterized an adduct formed by PhIP in vitro with the amino acid cysteine. This adduct should provide a biomarker of dietary PhIP exposure and potential prostate cancer risk that could be used to identify individuals for prevention and for monitoring the effect of chemoprevention strategies.

15. SUBJECT TERMS
Protein adduct, PhIP, biomarker, prostate cancer, Accelerator mass Spectrometry

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Introduction:

Prostate cancer is the second leading cause of cancer death in men in the United States, with African-Americans having the highest rate of prostate cancer in the world. Despite these statistics, the specific causes and risk factors for prostate cancer and reasons for the racial disparity remain elusive. A few epidemiology studies have indicated that exposure to PhIP, a rodent prostate carcinogen formed in meat during cooking, may be an important risk factor for prostate cancer in humans. There is also some evidence that African-American children have a 2-3 fold higher exposure to PhIP than White American children. Children are an important population to study because carcinogen exposure during development may lead to increased prostate cancer risk in later life. However, the epidemiology data are based upon PhIP exposure estimates from dietary questionnaires, rather than measurement of molecular markers that more accurately quantify an individual's internal dose and potential cancer risk. Therefore, a highly sensitive biomarker assay is urgently needed to clarify the role of PhIP in prostate cancer.

The goal of this project is to develop an assay that can be used to more accurately quantify human exposure to PhIP and potential prostate cancer risk. Our hypothesis is that an Accelerator Mass Spectrometry-based method can be developed to measure protein adducts of PhIP in the blood of humans. This will provide a measure of the internal dose, as well as the capacity for carcinogen bioactivation to a form that can initiate the cancer process. In a proof-of-principle study, we will use the assay to investigate the hypothesis that African-Americans may be at greater risk for prostate cancer than Whites because they have adduct levels that are 2-3 fold higher in childhood.

Our aims are to 1) Characterize the protein adducts formed by PhIP with the blood protein albumin. 2) Develop an ultrasensitive radiommunoassay for PhIP albumin adducts so that they can be assessed in populations of people. 3) Measure PhIP-albumin adduct levels in blood samples obtained from African-American and White male children.

Body:

During the second year of this grant (February 1, 2004 to January 31, 2005) we have continued to work on specific aim 1 of the proposal. In the first year we identified a cysteine adduct that was formed between N-acetoxy PhIP and a peptide, however prior to raising antibodies against this adduct, it is crucial that we establish if the adduct is formed in albumin *in vitro*, as well as with albumin *in vivo*. This task has required considerably more effort than anticipated due to the low levels of adducts formed *in vitro* with N-acetoxy-PhIP. The progress is described as follows:

Specific aim #1: Characterize the adducts formed with the blood protein albumin after exposure to PhIP.

The goal of this aim is to use *in vitro* methods to synthesize sufficient quantities of adducts for characterization by mass spectrometry and then establish if these adducts are formed *in vivo* in an animal model and humans.

Towards this goal, we have used 2 strategies to identify the adducts formed by PhIP. Firstly, we refined our methodology for cleaving adducts from proteins using acid hydrolysis. Heterocyclic amine adducts with a sulfur linkage to cysteine are cleaved by acid hydrolysis (Turesky *et al.*,

1987). Hence by treating the albumin samples with acid, recovering [^{14}C]PhIP through organic extraction and then analyzing using accelerator mass spectrometry (AMS), we can quantify the amount of the cysteine adduct formed in the rat and human plasma samples. Secondly, we have used 'traditional' mass spectrometry techniques to characterize other unknown albumin adducts formed *in vitro* and *in vivo* following exposure to unlabeled PhIP, deuterated PhIP and N-acetoxy PhIP.

1. ***Quantitation of cysteine adducts in human and rat albumin using acid hydrolysis and AMS:*** We investigated the effect of hydrolysis time, temperature and pH on acid hydrolysis efficiency. We concluded that 24hr is sufficient in achieving a complete or near complete hydrolysis of the acid labile bonds. Unexpectedly, 12M and 6M HCl acid treatments resulted in less than 6% recovery of PhIP while 1M, 0.1M and 0.01M HCl treatment resulted a consistent 17.4-18.1% recovery.

The methodology was then used to establish the percentage of PhIP-cysteine adduct in human and rat albumin samples. Twelve F344 male rats were gavaged either with corn oil only (controls) or 83.7mg PhIP in corn oil/kg body weight (dosed). Blood samples collected from the sacrificed rats were centrifuged at 3,000xg for 10min to obtain plasma. All samples are stored at -35°C until use. Albumin was isolated from the plasma as described in Dingley et al., 1999. Human albumin was isolated from the blood of two human subjects that had been administered 1μg/kg body-weight ^{14}C-PhIP. Samples were obtained both 8hr and 24hr post-PhIP exposure.

Our data shows a slightly higher level of acid labile adducts in rats, ranging from 20-34%, than in humans, where the acid labile adduct level ranged between 15-41% in the Albumin of 8hr samples and 8% in 24hr samples.

2. Characterization of PhIP-albumin adducts using mass spectrometry:
Albumin that had been modified with N-acetoxy PhIP and deuterated N-acetoxy PhIP *in vitro*, as well as albumin from rats dosed with PhIP, were analyzed by mass spectrometry to identify targets for modification.

All albumin samples were desalted and digested using Trypsin and Glu-C endopeptidases, following standard procedures.[1] Each set of peptide digestion consists of 4 samples: 2 control aliquots of unmodified albumin and 2 adduct aliquots of albumin-PHIP; one control and one adduct for trypsin digestion, the others for Glu-C digestion. Then all digested samples were completely dried using a speed vac.

Prior to injection into the mass spectrometer, they were re-dissolved (~10 pmol/μL) in 90% buffer A, 10% buffer B (buffer A: 10 mM ammonium acetate, buffer B: 90% acetonitrile and 0.1% formic acid; they are for nano-LC system). Data were collected on an FT-ICR mass spectrometer (Bruker Daltonics, MA) operated in the positive ion mode. The ionization source was operated under normal spray conditions with total flow rates of 100 nL/min. of nanospray. Typical conditions included a spray voltage of approximately 2 kV and a drying gas capillary temperature of 350 ^{0}C. Each dataset consisted of 400 experiments of 8 scans/experiment with a duty cycle time of 6 sec/experiment.

Bruker Xmass software was used to control the mass spectrometer. Software used for data analysis was provided by Dr. Gary H. Kruppa, Vice President of Bruker, Inc.
The flow chart of experimental and analytical methods is shown in Figure 1. Data collected on the control samples were compared to *in silica* digested albumin (from the protein database at Swiss-Prot/TrEMBL, www. expasy.org) to validate the peptide digestion and mass spectrometric methods. To be eligible, the experimental mass list should recover at least 95% fragments shown in that of *in silica* data. Next, data collected on the adducts were compared to *in silica* digested albumin with the addition of the mass of PhIP molecule and $\Delta m \leq 0.15$. The adduct fragments were selected if their mass does not coexist with those of the control. Finally, the putative albumin-PhIP adduct fragments were validated by their presence in both the Tryptic- and the Glu-C-digested adduct samples.

***a)* Human serum albumin (HSA) reacted with N-acetoxy PhIP**
The mass list for tryptic-digested HSA-PhIP sample displayed 2,799 values, among which 6 lead fragments were identified. Similarly, 6 lead fragments were found from the 4,312 mass values listed for Glu-C-digested HSA-PhIP sample. These putative peptide fragments are shown in Table 1. Comparison of the tryptic-digested lead fragments with those of Glu-C-digested yielded the unique adduct fragment of TYVPK (residues 520-524 at the N-terminal of HSA sequence). Figure 2 displays the overall 2D mass spectrum for tryptic-digested HSA-PhIP, as well as the 1D trace of the putative lead fragment (residues 509-524). As indicated in the literature, Cysteine, Methionine, Tyrosine, or even Tryptophan are possible binding sites for heterocyclic amines such as PhIP.[2-5] Thus, these results suggest that PhIP binds to residue Tyrosine 521 in HSA. Figure 3 shows crystal structure of HSA with the PhIP binding sequence (red color) mapping on the protein surface.

***b)* Human serum albumin reacted with deuterated (D3) N-acetoxy PhIP**
The adduction in this sample was carried out with HSA and a mixture of N-Acetoxy- PhIP and N-Acetoxy-PhIP-D3 (about 1:1 by weight). In addition, there were 2 separate samples incubated in 2h and 24h to assess the correlation between adduction level and incubation time.

Due to the presence of both regular and deuterated PhIP, analysis of this type of sample was conducted differently than it was for the regular ones. The fragment leads in each enzyme-digested sample were selected according to the criteria: (1) $\Delta m \leq 0.15$ for the experimental mass relative to the *in silica* one, (2) $\Delta m = 3.0186$ for the peptide mass of the adduct containing PhIP-D3 relative to that with regular PhIP, (3) co-elution or close elution time between the fragment containing PhIP-D3 relative to that with regular PhIP, (4) the same isotopic pattern or multiplicity for signals containing PhIP-D3 relative to that with regular PhIP.

Data on the *in vitro* HSA-PhIP(D:H) sample with 24h incubation time yielded 3,432 fragment masses, 8 of which met criteria (1) and (2). Two out of 8 candidates satisfied criterium (3) on the elution time. But none of them have the same isotopic pattern. Hence, no adduct fragment was selected for this sample.

Data on the *in vitro* HSA-PhIP(D:H) sample with 2h incubation time gave 1,233 peptide masses,

among which 4 met criteria (1) and (2). Only 1 of these 4 candidates made to condition (4). Figure 4 depicts the similarity in isotopic pattern for the adduct signals of HSA-PhIP(H3) (top) and HSA-PhIP(D3) (bottom) of the peptide fragment AACLLPK (sequence position 199-205). However, their elution times are about 1.5 min. apart from each other. And due to the stronger hydrophobicity of deuterium relative to proton, it was unexpected that the deuterated fragment eluted before the corresponding protonated fragment. Thus, no fragment could be selected. However, this discussion is based only on the tryptic-digested sample. Data on the Glu-C digested *in vitro* HSA-PhIP(D:H) will be done and discussed.

It should be noted that the spectrometer was so sensitive in terms of being able to reflect the 3-fold peak intensity of the adduct fragment containing PhIP(D3) relative to that with regular PhIP (which is 3.0186 unit mass lower than its deuterated correspondent). Also it should be noted that the fragment leads yielded from these two samples of the *in vitro* HSA-PhIP(D:H) were not identical, although they underwent the adduction and later the enzymatic digestion in the same conditions, at the same time, with only different incubation times.

***c)* Rat serum albumin from animals dosed with [^{14}C]PhIP**

Of the 7,354 mass values from data on the tryptic-digested RSA-PhIP(^{14}C), 10 fragment leads were identified. Similarly, 9 leads were found from 3,932 mass values for data on the Glu-C-digested RSA-PhIP(^{14}C). All the leads are listed in Table 3. Combination of the leads in both tryptic- and Glu-C-digested data resulted in the following adduct fragments:

Sequence	Position
SEIAHR	29-34
EAHKSEIAHRFK	25-36
DLGE	37-40
TCVADENAE	76-84
LADCCAK	111-117
AACLTPK	199-205
AACLTPKLDAVKEK	199-212
EVCKNYAE	338-345
GPNLVARSK	595-604

According to the molecular simulated structure of RSA based on crystal structure of HSA, all the Cysteine residues in the above sequences are involved in disulfide bonds. Unless these bonds are broken, the sulfhydryl group in these Cysteines cannot bind the PhIP molecule. Also these results suggested that beside Cysteine (C), Methionine (M), Tyrosine (Y), Tryptophan (W) as indicated in the literature,[2-5] PhIP possibly binds to other electron-rich side chain residues such as Glutamate (E), Aspartate (D), Lysine (K), Arginine (R), Serine (S), Threonine (T).

Discussion:

An overview of all the work discussed above suggests that the nanoLC nanospray FTMS is sufficiently sensitive to search for very low level of PhIP adducts in proteins. Also the adducts formed between PhIP and Cysteine are just one of the products and PhIP may bind to other amino acid residues as well.

Results deduced from *in vitro* HSA-PhIP and *in vivo* RSA-PhIP do not confirm the binding of PhIP to the only Cysteine with the free sulhydryl group in this protein. Crystal structure of HSA shows that the sulfhydryl side chain of this Cysteine is toward the interior of the protein, preventing it from readily coupling with the external ligand.[7]

The most sensitive mass spectrometer has been used for this study. Considering the peptide-PhIP sample as an external standard, this spectrometer proved its ability to detect adducts at very low levels. So, if albumin-PhIP adducts were formed, this mass spectrometric system should succeed in picking up the adducts. Another proof of the sensitivity of this instrument is the variety of adduct fragments yielded from the high dose *in vivo* RSA-PhIP(^{14}C) shown in the Results section, and their absence from the impure sample of *in vivo* HSA-PhIP(^{14}C). Also an evidence of the instrumental sensitivity is the 3-fold more intense of the peptide signal containing PhIP-D3 compared to its corresponding with regular PhIP (Figure 4) in the mass spectrum for *in vivo* HSA-PhIP(D:H).

However, the work on protein-PhIP adducts has still been inconclusive, in terms of the effective binding of PhIP to serum albumin. Current results suggest the presence of multiple protein-PhIP adducts and it seems that PhIP non-specifically binds to a variety of residual side chains at different locations. The difference in fragment leads found in the 2 samples of *in vivo* HSA-PhIP(D:H), which only differ from each other by the incubation time, speaks strongly for the random binding of PhIP on serum albumin. In addition, although data on Glu-C-digested *in vivo* HSA-PhIP(D:H) have not been completed, the spreading of PhIP and PhIP-D3 on the tryptic-digested sample and the absence of a specific binding site, where both fragment signals containing PhIP and PhIP(D3) coexist, also suggest the non-specific binding of PhIP to serum albumin.

Key Research Accomplishments:

During the second year of this grant, we have shown that:

- Data indicates that the cysteine adduct is formed *in vivo* in rats and humans exposed to PhIP. The level of these adducts is higher in rats than humans.
- The adducts formed between PhIP and Cysteine are just one of the products and PhIP may bind to other amino acid residues as well.

Reportable Outcomes:

Presentations

Ahn, S., Ubick, E. and Dingley, K. Characterization of the protein adducts formed by the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in humans. Genetic and Environmental Toxicology Association of Northern California, 7th May, 2004, Oakland, CA. **Sylvia won first prize for this poster in the poster competition.**

Ahn, S., Ubick, E. and Dingley, K. Characterization of the protein adducts formed by the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in humans. 6th Annual UC Davis Conference for Environmental Health Scientists, August 30, 2004, The Embassy Suites, Napa, California.

Dingley, K.H. Ultra-sensitive quantitation of Heterocyclic amine adducts using accelerator mass spectrometry: from risk identification to chemoprevention'. United Kingdom Environmental Mutagen Society, 27th Annual Meeting, University of Loughborough, July 4-7, 2004.

Dingley, K.H. 'Quantitation of macromolecular targets of heterocyclic amine carcinogens at low dose'. 228th American Chemical Society National Meeting, Philadelphia, PA, August 22-26, 2004.

Dingley, K.H. 'Ultra-sensitive quantitation of heterocyclic amine adducts using accelerator mass spectrometry: from risk identification to chemoprevention'. Tenth Annual Cancer Research Symposium, UC Davis Cancer Center, October 20-21, 2004.

Tran, A.-T. T., Young, N.L., Ahn, S.J., Dingley, K.H. Structural Characterization of DNA-MeIQx adducts. 10th Annual Cancer Research Symposium, 10/20/04 – 10/21/04, UC Davis Cancer Center, Sacramento, California.

Tran, A.-T. T., Young, N.L., Palmblad, M., Ahn, S.J., Dingley, K.H. Structural Determination of the Adducts formed by Heterocyclic Amines with Biomacromolecules. 6th Annual UC Davis Conference for Environmental Health Scientists, 8/30/04, The Embassy Suites, Napa, California.

Tran, A.-T. T., Young, N.L., Ahn, S.J., Butler, B.K., Lebrilla, C.B., Benner, W.H., Cosman, M., Maxwell, R.S., Dingley, K.H. Structural Determination of the Adducts formed by Heterocyclic Amines with Biomacromolecules. CMS Postdoctoral Symposium 2004, 7/27/04, Lawrence Livermore National Laboratory, Livermore, California.

Employment/Research Opportunities

Over the period of this grant, we have supported a post-doctoral fellow (Anh-Tuyet Tran) and a biomedical scientist (Sylvia Ahn), to work on this project. This was the first opportunity for Ahn and Sylvia to work in prostate cancer research.

Conclusions:

The observed formation of PhIP-cysteine adducts in vitro and in vivo justify the continued development of an assay that can quantitate these adduct levels in humans. The ultimate question of whether or not these adducts can serve as markers of prostate cancer susceptibility, initiation or progression can only be answered with such an assay. Over the next year we will focus efforts on synthesizing a peptide with the major PhIP-cystein adduct as a substrate for raising monoclonal antibodies in mice, and the use of those proteins to develop an AMS-based assay that is sensitive enough to detect the adducts in archived samples from humans dosed with low amounts of 14C-labeled PhIP.

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